

**STUDIES ON THE FLUORIMETRIC
DETERMINATION OF SUBNANOMOLE
AMOUNTS OF AMMONIUM COMPOUNDS BY
ION-PAIR EXTRACTION WITH
APPLICATION TO BIOANALYSIS**

by
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WITH APPLICATION TO BIOANALYSIS

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TO MY WIFE AND DAUGHTER,
KERSTIN AND ULRIKA

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List of publications

In this survey the following papers will be discussed and referred to by Roman numerals.

- I. D. Westerlund and K. O. Borg. Fluorimetric determinations by ion pair extraction. Part 1. Extraction of ammonium compounds as ion pairs with anthracene-2-sulphonate. Acta Pharm. Suecica 7, 267 (1970)
- II. D. Westerlund, K. O. Borg and P.-O. Lagerström. Fluorimetric determinations by ion pair extraction. Part 3. Extraction constants of ion pairs between anthracene-2-sulphonate and mono- and divalent amines. Acta Pharm. Suecica 9, 47 (1972)
- III. P.-O. Lagerström, K. O. Borg and D. Westerlund. Fluorimetric determinations by ion pair extraction. Part 4. Studies on quantitative ion pair extraction of ammonium compounds with anthracene-2-sulphonate as counter ion. Acta Pharm. Suecica 9, 53 (1972)
- IV. D. Westerlund and K. O. Borg. Fluorimetric determinations by ion pair extraction. Part 5. Studies on ion pair extraction with the fluorescent anion 9,10-dimethoxyanthracene-2-sulphonate. Anal. Chim. Acta 67, 89 (1973)
- V. D. Westerlund. Fluorimetric determinations by ion pair extraction. Part 6. Blanks of 9,10-dimethoxyanthracene-2-sulphonate in extractions to methylene chloride + 1-pentanol. Acta Pharm. Suecica 11, 581 (1974)

- VI. D. Westerlund and K. H. Karset. Fluorimetric determination of propantheline in human blood plasma by an ion-pair extraction method. *Anal. Chim. Acta* 67, 99 (1973)
- VII. D. Westerlund. Extraction of subnanomole amounts of amines and quaternary ammonium compounds from albumin solutions and human blood serum. *Acta Pharm. Suecica*. In press.
- VIII. D. Westerlund and A. Söderqvist. Distribution of palmitic acid between aqueous solutions and methylene chloride. *Acta Pharm. Suecica*. In press.

Symbols and abbreviations

The following symbols and abbreviations, undefined in the text, are used:

Q = cation component in the aqueous phase (the symbol HA is also used)

X = anion component in the aqueous phase

QX_{org} = ion pair extracted to the organic phase

C^0 = initial concentrations

$C'_Q(X)_{org}$ = total concentration of Q extracted to the organic phase as ion pair with X

$$C'_Q(X) = C_Q^0 - C'_Q(X)_{org}$$

$$C'_X(Q) = C_X^0 - C'_X(Q)_{org}$$

$$\alpha_{QX} = C'_Q(X)_{org} / [QX]_{org}$$

$$\alpha_Q(X) = C'_Q(X) / [Q]$$

$$\alpha_X(Q) = C'_X(Q) / [X]$$

$$k_{diss}(QX) = [Q]_{org} \times [X]_{org} / [QX]_{org} = \text{dissociation constant of } QX \text{ in organic phase}$$

$$k_d(A) = [A]_{org} / [A] = \text{partition coefficient of A}$$

$$k_d(HX) = [HX]_{org} / [HX] = \text{partition coefficient of HX}$$

$$K'_{HX} = a_{H^+} \times [X] / [HX] = \text{apparent acid dissociation constant}$$

AS = anthracene-2-sulphonate

MAS = 9,10-dimethoxyanthracene-2-sulphonate

PT = protriptyline

NPT = N-methylprotriptyline

MPT = N,N-dimethylprotriptyline

TBA = tetrabutylammonium

TBAH = tetrabutylammonium hydroxide

Introduction

In recent years the rapid development of the analytical pharmaceutical chemistry has contributed to a deeper knowledge of the absorption, metabolism and excretion of drugs and metabolites in the human body. Samples of biological origin often have a complex nature and the drug is mostly present in a very low concentration. Due to these facts, good selectivity and high sensitivity of the analytical method are of utmost importance.

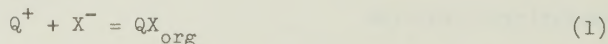
An extraction step is, as a rule, necessary in the analytical procedure. The extraction must be based on a principle that gives good selectivity and also possibilities to a regulation of the extraction degree. Such properties are found in the ion-pair extraction methods which can be used on drugs and metabolites that have an ionized group.

Ion-pair extraction procedures can also be used to increase the sensitivity in the quantitation process. Potent drugs are often present only in pico- or nanogram amounts in the biological sample, and fluorimetry is one of the few techniques that gives the required sensitivity. However, few substances give a fluorescence of sufficient intensity, but the necessary high sensitivity can be obtained, even for non-fluorescent substances, by coupling the object to an intensely fluorescent counter ion by an ion-pair extraction process.

Conditions for ion-pair extraction

Basic theory

The principles of ion-pair extraction are given by the following net reaction:



and the equilibrium constant of the process is defined by:

$$E_{QX} = [QX]_{org} \times [Q]^{-1} \times [X]^{-1} \quad (2)$$

The main reaction (1) is often influenced by different kinds of side-reactions, e.g. protolysis, associations and dissociations. It is then convenient to use a conditional extraction constant defined by

$$E_{QX}^* = C'_{Q(X)org} \times (C'_{Q(X)} \times C'_{X(Q)})^{-1} \quad (3)$$

E_{QX}^* can be related to E_{QX} by means of α -coefficients [1]

$$E_{QX}^* = E_{QX} \times \alpha_{QX} \times (\alpha_{Q(X)} \times \alpha_{X(Q)})^{-1} \quad (4)$$

The α -coefficients increase with increasing extent of the side-reactions, and they are equal to unity when the influence of side-reactions is negligible.

Determination of constants

Calculations of adequate conditions for selective and quantitative ion-pair extractions are preferably based on stoichiometric or conditional constants for the equilibrium processes in the system.

Determination of the extraction constants and the constants for side-reactions was performed according to the principles given in refs. [2,3]. The constants were evaluated by slope analysis.

The standard deviations of the extraction and dissociation constants are given in papers II, IV and VI and were calculated by the method of least squares, assuming that the errors in the measurements are relative and normally distributed.

Magnitude of the constants

1 + 1 ion pairs
.....

The magnitude of the extraction constants depends on the structure of the anion and the cation and the nature of the organic phase. Extraction constants of TBA with some hydrophobic anions are given in Table 1 (organic solvent: methylene chloride). The two anthracene-sulphonates have about ten times lower stoichiometric constants than

T A B L E 1

Extraction constants of tetrabutylammonium ion pairs

Organic phase: methylene chloride

Aqueous phase: phosphate buffers ($\mu = 0.1$)

Anion	$\log E_{QX}$
Anthracene-2-sulphonate	5.45
Dimethoxyanthracene-2-sulphonate	5.85
Bromothymol blue	9.31 ^a
Picrate	6.68 ^b
Perchlorate	4.64 ^c

a: ref. [9]

b: ref. [5]

c: ref. [4]

picrate and more than 1000 times lower constants than the monovalent anion of bromothymol blue. The partition of picrate and bromothymol blue as acids to methylene chloride is, however, so large that it limits their use in quantitative extractions from acidic aqueous phases due to high blanks. The aprotic sulphonates give blanks which are independent of pH.

AS and MAS give, with symmetrical quaternary ammonium compounds, dissociation constants of the order $10^{-4.4} - 10^{-5.0}$ in methylene chloride (I, III, V), which is in accordance with earlier results obtained with inorganic [4] as well as organic [5] anions. The dissociation constants with tertiary and secondary amines are about 100 to 1000 times lower, probably due to the fact that hydrogen bonding within the ion pair increases its stability [6,7]. The difference is larger than for corresponding ion pairs of picrate [5] and inorganic anions [8]. Unsymmetrical quaternary ammonium ions with aromatic substituents also give considerably smaller dissociation constants than symmetrical (VI, VII).

1 + 2 ion pairs
.....

The extraction of emetine as 1 + 1 and 1 + 2 ion pair with AS was studied in paper II. Since the extraction constants for the two ion pairs are defined in different ways, their magnitude cannot be directly compared. A comparison can be based on the partition ratios

$$D_{HA}(X) = E_{HAX} \times [X] \quad (1 + 1 \text{ ion pair}) \quad (5)$$

$$D_{H_2A}(X) = E_{H_2AX_2} \times [X]^2 \quad (1 + 2 \text{ ion pair}) \quad (6)$$

Quantitative extraction of emetine as H_2AX_2 ($D = 100$) requires about 64 times higher concentration of AS than extraction as HAX. If a more hydrophobic anion is used, the difference is less, since the properties of the ion pair is then dominated by the anion (cf., bromothymol blue [9]).

Organic solvent

Halogenated hydrocarbons, e.g. chloroform and methylene chloride, are, as a rule, used by extraction of hydrophobic ion pairs. For more hydrophilic ion pairs, it is often necessary to add a stronger solvating agent, e.g. an alcohol, but a drawback is the simultaneous decrease of the selectivity [10].

The acid partition of MAS with methylene chloride + n-pentanol as the organic phase was studied in paper V. The partition data are presented as an ion-pair extraction of HMAS, which facilitates the comparison of the extraction properties of H^+ and other cations.

It can be shown that

$$E_{HMAS} = f_{H^+} \times k_d(HMAS) / K'_{HMAS} \quad (7)$$

where f_{H^+} is the activity coefficient of H^+ .

Pentanol has a drastic effect on the extraction of HMAS: addition of 1% increases E_{HMAS} about 11 times while addition of 10% gives an increase of 9000 times. The increase of the extraction constants with other cations, e.g. TBA (I) and MPT [11], is considerably less: about four times for an addition of 10% alcohol.

The difference between extraction constants of AS and MAS ion pairs is about equal in pure halogenated hydrocarbons (I,II,IV) and in mixtures thereof with pentanol (I,V), in spite of the presence of two hydrogen-accepting methoxy-groups in MAS.

HMAS is solvated with two or three pentanol molecules, depending on the concentration of the alcohol (V). In view of the discussion above it is probable that the pentanol molecules are bound to the hydrated proton (cf., [12,13]).

Fluorescence measurements

Fluorimetry can be used to measure extremely low concentrations of substances with suitable structures, present in an appropriate environment. A review of the factors controlling fluorescence intensities and wavelengths is given in ref. [14]. Highly conjugated aromatic compounds usually emit the most intense fluorescence which is increased by electron-donating substituents and decreased by electron-attracting. The presence of a heteroatom in the aromatic system makes the fluorescence properties of the molecule more susceptible to changes in the environment.

Very few substances possess all the properties required for an intense fluorescence, but a derivatization process can often transfer the object into an appropriate form. High fluorescence can be obtained by increasing the degree of conjugation [15], by formation of a heterocycle [16], or by coupling the object with a fluorescent molecule [17]. Enzymatic reactions have also been used in quantitative determinations of non-fluorescent compounds either by formation of fluorescent coenzymes [18] or by transformation of enzymatic substrates into fluorescent form [19]. In this thesis, quantitative fluorimetric determinations based on ion-pair extractions with fluorescent counter ions, are used.

The fluorescence intensity depends on the apparatus (instrumental response and the intensity of the emitted light) and on the object (concentration, molar absorptivity (ϵ) and fluorescence yield (ϕ)) [20].

Since most fluorescence measurements utilize only a part of the emission spectrum, a compensation of the shape of the spectrum is also necessary. An expression for the fluorescence sensitivity, which is independent of the apparatus, is then given by $\epsilon \times \phi / H$, where H is the half-band width of the emission spectrum [21].

The fluorescence properties of the anthracene derivatives and their ion pairs with TBA are summarized in Table 2. For comparison the properties of quinine is also included. A remarkable fact is the different influence of methylene chloride on the fluorescence of the reagents; the fluorescence yield is higher in the organic phase than in the aqueous for MAS, while the opposite is true for AS.

T A B L E 2

Fluorescence yield and sensitivity

Substance	Excitation maximum (nm)	Emission maximum (nm)	Fluorescence yield = ϕ	Fluorescence sensitivity = $\log \epsilon \phi / H$
Solvent: water				
NaAS	257	415	0.40	5.22
NaAS	359	415	0.40	3.76
NaMAS	263	479	0.36	5.11
NaMAS	383	479	0.36	3.71
Quinine	345	460	0.55	3.88
Solvent: methylene chloride				
TBA-AS	260	410	0.10	4.74
TBA-AS	359	410	0.10	3.22
TBA-MAS	267	452	0.69	5.45
TBA-MAS	383	452	0.69	4.05

Corrected wavelengths for excitation and emission maxima.

In conclusion the fluorescence intensities obtained are very high and constitute a convenient starting-point for the construction of sensitive analytical methods (IV).

Quantitative determinations in aqueous solutions

Hydrophobic sulphonates are often very useful as reagents in quantitative extractions since they give high extraction constants and can be used in a large pH-range. In extractions to halogenated hydrocarbons they also give low blanks (I,IV) which is advantageous in developing sensitive analytical methods.

When extracting substances in low concentrations ($< 10^{-6}$ M), it is of ultimate importance that the solvents and reagents are of high purity [22] since impurities, even in trace amounts, can affect the distribution of the object [23]. Co-extracted components can have influence on equilibria in the organic phase (V).

Adsorption to the surface of the glass equipment [24,25] can give rise to serious losses of objects present in low concentrations. The recoveries of MPT, NPT and PT in extractions from aqueous solutions depend on the following: (a) technique of organic phase transfer; (b) presence of adsorption inhibitor (e.g. alcohol) in the receiving vessel; (c) ionic strength of the aqueous phase; (d) treatment of the glass equipment (e.g. silanization) (VII).

Fluorimetric standard curves

The fluorescence intensity of an ion pair, where only one of the components is fluorescent, may be affected by the properties of the other ion (II). By addition of TBAH (10^{-3} M) all AS and MAS ion pairs can be transformed into the TBA ion pair, and the same standard curve can be used for all ion pairs, irrespective of the cation.

By determination of amines in the organic phase, TBAH (10^{-3} M) is also added to obtain the fluorescence of the base form in all cases.

Quantitative extractions with AS and MAS

TBA and amitriptyline were used as model substances for investigations of the properties of AS and MAS as counter ions in quantitative extractions in the low concentration range (III,IV).

The fluorimetric measurements were made either in the organic phase or after a quantitative back-extraction of the fluorescent anions to an aqueous phase.

The quantitation is based on calibration curves prepared in two different ways. The first ("standard curve") is made from known solutions (about 10^{-5} M) of the anions (aqueous phase) or their ion pairs (organic phase) which are diluted and measured fluorimetrically. The second type ("reference solution") is made from appropriately diluted aqueous solutions which are extracted as the samples.

Quantitative recoveries were obtained in all cases when the quantitation was made by the "reference solution" method. When the "standard curve" method was used, extractions with AS gave, in most cases, lower recoveries, while MAS extractions were almost quantitative. The low recoveries with AS were obtained at sample (cation) concentrations below 10^{-6} M. Extractions of AS in $< 10^{-6}$ M solutions with an excess of the cation were, however, quantitative.

Blanks

The limit of determination depends on the standard deviation (s) of the blank and also on the magnitude of the blank since the s usually increases with an increasing blank signal [26]. In fluorimetric measurements based on ion-pair extractions it is favourable to use halogenated hydrocarbons as organic phases since they give low blanks, due to a low tendency to extract the reagents as ion pairs with buffer components.

Addition of a lipophilic alcohol to the organic phase increases the extraction of acidic reagents (V). The influence of pentanol in the extraction of MAS as ion pair with H^+ is illustrated in Fig. 1. Even the addition of a small amount of alcohol has a large effect on the distribution of MAS. Similar observations were made by extractions of AS ion pairs to chloroform + pentanol in paper I.

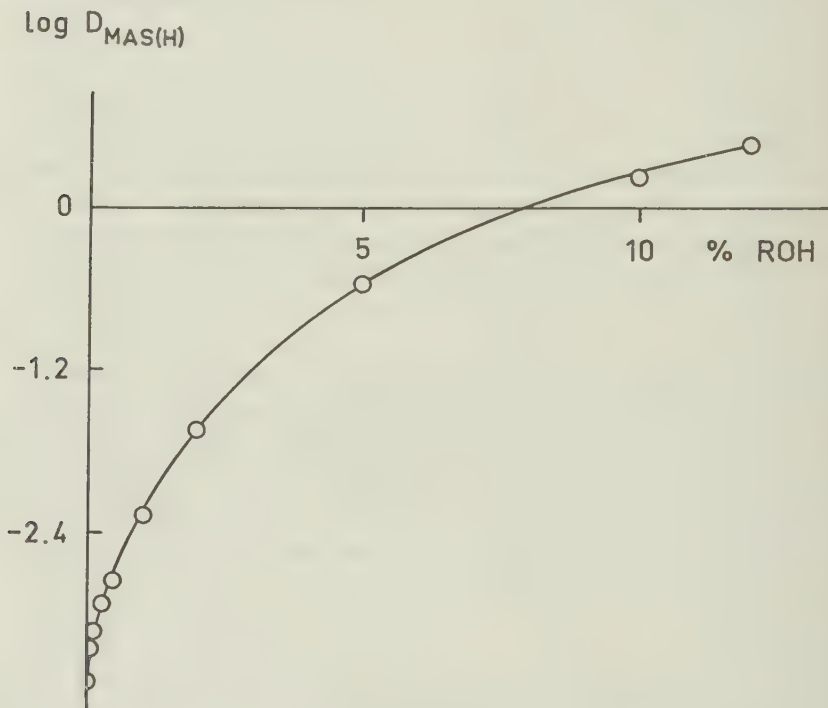


FIGURE 1

Partition of 9,10-dimethoxyanthracene-2-sulphonic acid

Organic phase: methylene chloride + n-pentanol (ROH)

Aqueous phase: 0.1 M $HClO_4$

Other components of the aqueous phase can also have influence on the magnitude of the blank. By extraction of HMAS from 0.1 M solutions of HCl , HNO_3 and HClO_4 , co-extraction of the inorganic acids affects the extraction of HMAS, due to their influence on the dissociation of HMAS in the organic phase. The lowest extraction is obtained from HClO_4 and the highest from HCl .

Incomplete blank compensations can result in serious errors. Thus, it is possible that the low recoveries obtained by extraction with AS (III) may be due to the use of over-compensating blanks given by e.g. impurities in the reagent solution. The distribution ratios of an anion (X) as ion pair with an impurity (B) in blank and sample (Q) are given by:

in the blank
.....

$$D_{X(B)} = E_{BX} \times C_B^0 + (C_X^0)^{-1/2} \times (E_{BX} \times k_{\text{diss}(BX)} \times C_B^0)^{1/2} \quad (8)$$

in the sample
.....

$$D_{X(B)} = E_{BX} \times C_B^0 + (C_X^0)^{-1/2} \times E_{BX} \times k_{\text{diss}(BX)} \times C_B^0 \times (E_{BX} \times k_{\text{diss}(BX)} \times C_B^0 + E_{QX} \times k_{\text{diss}(QX)} \times [Q])^{1/2} \quad (9)$$

It is assumed that B and X are present in excess to Q.

The combination of equations (8) and (9) gives

$$\begin{aligned} \frac{(D_{X(B)} - E_{BX} \times C_B^0)_{\text{blank}}}{(D_{X(B)} - E_{BX} \times C_B^0)_{\text{sample}}} &= \\ &= \frac{(E_{BX} \times k_{\text{diss}(BX)} \times C_B^0 + E_{QX} \times k_{\text{diss}(QX)} \times [Q])^{1/2}}{(E_{BX} \times k_{\text{diss}(BX)} \times C_B^0)^{1/2}} \quad (10) \end{aligned}$$

If $E_{QX} \times k_{\text{diss}(QX)} \times [Q]$ is not negligible, compared to $E_{BX} \times k_{\text{diss}(BX)} \times C_B^0$, an error is introduced by the blank compensation, and too low recoveries are obtained.

Determinations in biological samples

Determinations in biological material can offer large problems due to the complex nature of the sample. The object is mostly present in a very small amount, and many sample components have much higher concentrations. Some components, e.g. proteins, also have the tendency to bind the object as a complex. Furthermore, ionic components can give rise to disturbing ion-pair extractions in the organic phase and/or ion-pair formation in the aqueous phase.

Protein binding

In blood many substances are bound to proteins, mostly albumin [27] but also to lipoproteins [28,29]. A vast number of publications concerning plasma protein binding has appeared in the literature and some reviews have also been published [30,31]. The binding of anions has been thoroughly studied, and seemingly, the anions are more extensively bound than cations and uncharged molecules of corresponding size [32].

The conformation and charge of serum albumin changes with pH and very large structural changes appear at pH below 4 and above 10 [33]. Consequently, protein binding is strongly pH-dependent; the binding of phenothiazines [34] and sulfonamides [35] to serum albumin is reported to decrease with decreasing pH. In extractions of biological samples the presence of an organic solvent may affect the conformations of proteins (cf., [36]) and consequently the binding of the object.

The influence of protein binding on extractions is illustrated by eq. (11) (VII), which is valid when the concentration of the object, A, is low and the protein is present in such large excess that a 1 + 1 complex will dominate.

$$D_A = k_d(A) \times (1 + K_{PA} \times [P])^{-1} \quad (11)$$

where

A = amine

K_{PA} = equilibrium constant for a 1 + 1 complex

[P] = concentration of the protein.

The concentration of serum albumin (the most abundant protein) in blood is normally $(4.1 - 6.5) \times 10^{-4}$ M, and consequently K_{PA} must be $> 5 \times 10^3$ to give a reduction of D_A of $> 10\%$. $K_{PA} \approx 10^4$ has been reported for the binding of some amines (thymoleptic drugs) to serum albumin at pH 7.4 [37]. Results from extractions of PT and NPT from albumin solutions also indicate the presence of binding (VII).

Reductions in extraction efficiency by protein binding can be avoided by dilution of the sample or by precipitation of the protein. However, the presence of a precipitate may be accompanied by a decreased recovery depending on adsorption or occlusion of the object, which is observed by the determination of propantheline in blood plasma (VI).

Precipitates can also appear in extractions as a result of the denaturing effect of the organic solvent on the protein. Halogenated hydrocarbons often cause heavier precipitates than, e.g. aliphatic and aromatic hydrocarbons, which to some extent limit their use.

Anions in blood

Numerous anions are present in blood [38] and chloride and bicarbonate have such concentrations that they can give rise to ion-pair extractions of hydrophobic amines and quaternary ammonium compounds as shown in the extractions of PT, NPT and MPT from albumin and serum solutions in paper VII.

The fatty acids ($C_{12} - C_{22}$) are the most hydrophobic organic acids present in blood and give rather high extraction constants with cations. Palmitic acid, which is about the intermediate in hydrophobicity of the acids, was chosen as a model compound for distribution studies (VII, VIII).

The distribution ratio of an aprotic cation (Q) with a fatty acid is given by

$$D_{Q(A)} = E_{QA}^* \times C_{A(Q)}' = E_{QA} \times (\alpha_{A(Q)})^{-1} \times C_{A(Q)}' \quad (12)$$

where $\alpha_{A(Q)}$ compensates for the protolysis and acid partition of A. At $pH \geq 7$ and for straight chain fatty acids with ≥ 8 alkyl carbons, $D_{Q(A)}$ gets the form

$$D_{Q(A)} = E_{QA} \times K_{HA}' \times (a_{H^+} \times k_{d(HA)})^{-1} \times C_{A(Q)}' \quad (13)$$

The extraction constants increase with increasing number of alkyl carbons (about 0.6 log units/ CH_2), but the acid partition increases with the same rate (myristic acid has $\log k_{d(HA)} / K_{HA}' = 10.6$ [39] and palmitic acid, 11.8 (VIII)). This means that the quotient, $E_{QA} / k_{d(HA)}$, in eq. (13) is constant, and consequently the extracting ability of all fatty acids with ≥ 8 alkyl carbons is equal in ion-pair extractions at $pH \geq 7$.

By extractions of cations with fatty acids from blood serum and plasma the conditional extraction constants are further decreased by protein binding of the anion [40].

In distribution studies of palmitic acid between aqueous buffers and methylene chloride an adsorption of the palmitate ion to the interface between the liquids is observed at concentrations $> 8 \times 10^{-8}$ M in the aqueous phase. The critical micelle concentration in the buffer solutions is very low and a visible precipitate at the interface is obtained for aqueous phase concentrations $> 2.4 \times 10^{-5}$ M (VIII).

Extractions of amines and quaternary ammonium compounds from albumin solutions and human blood serum

The distribution of MPT, NPT and PT between albumin and serum solutions and methylene chloride was studied in the concentration range (5×10^{-9} - 10^{-4} M) (VII).

Addition of albumin to the aqueous phase increases the distribution ratios of both MPT and PT, which is probably due to anionic impurities in the albumin capable of giving extractable ion pairs with the ammonium ions.

Addition of serum gives an even higher extraction increase which is dependent on both pH and the serum concentration. The extraction degree of MPT increases with pH to a maximum at pH 8-9 and decreases at higher pH. A weak acid in the serum, which is bound to the serum proteins in anionic form might be responsible for the effects.

Addition of albumin to solutions of NPT decreases the distribution ratio, indicating that the tertiary amine is harder bound to the protein than the secondary and quaternary compounds.

Conclusions

The starting-point in the computation of conditions for extractions from blood plasma and serum is often extraction data obtained from pure aqueous solutions. The results presented here indicate that components in the biological material may give rise to deviations from the calculated extraction conditions: extraction increase or decrease may appear depending on the structure of the object. The selectivity may thus be different in pure aqueous solutions and in the presence of biological material.

The concentration of chloride in blood is so large (0.1 M) that it decreases the conditional extraction constants of other ion-pair systems. The effective concentration of a cationic reagent is decreased by chloride ion-pair extraction, while the extracting

ability of an anionic reagent is decreased by the competitive extraction of the object as chloride ion pair. It is necessary to use a higher concentration of the reagent to compensate for these effects.

Determination of propantheline in blood plasma

The fluorescent anion, MAS, was used in a determination of the anticholinergic drug, propantheline (a quaternary ammonium compound), in blood plasma.

Propantheline is extracted from acidified plasma to methylene chloride as ion pair with perchlorate. The organic phase is then purified by a re-extraction with an alkaline aqueous phase, containing perchlorate in such a concentration that the propantheline is retained in the organic phase as ion pair. In this way the hydrolysis of the ester function in propantheline is prevented. In the final extraction step a quantitative exchange between perchlorate and the fluorescent MAS is obtained by equilibration with an aqueous phase containing an adequate concentration of MAS. Finally, the concentration of the ion pair is determined fluorimetrically.

The recoveries from plasma are strongly dependent on pH, 0% at pH 8.4 and 90% at pH 3.7, which is probably due to hydrolysis and/or a pH-dependent protein binding of propantheline. A large excess of perchlorate (about 2000 times the theoretical concentration) is needed to give a satisfactory extraction degree of the drug from the plasma. The use of such a high reagent concentration has the drawback of giving rise to co-extractions of other cations which have to be removed by purification steps. 13 ng/ml of propantheline can be determined with a precision of 4.6% (s.) and a recovery of 80%.

Blanks and limit of detection

The blanks, obtained from albumin and serum solutions (VII), are independent of pH but increase with the concentration of albumin. The standard deviations are 10-20% of the mean blank. The limit of determination, $C_{\min}$, [26], defined as the smallest concentration that can be determined with a relative standard deviation of 5%, is, in paper VII, calculated by:

$$C_{\min} = 28.2 \times s_c \times n^{-1/2} \quad (14)$$

where

s_c = standard deviation (concentration units)

n = number of determinations (equal in sample and blank).

Eq. (14) is valid under the assumption that the blank and sample can be determined with the same standard deviation. In some cases this may be an invalid approximation, and a more reliable figure of sensitivity of the analytical method may be the limit of detection. The statistical problems connected with the limit of detection have been extensively treated by H. Kaiser [41-46].

In fluorimetric measurements the analytical signal (F) is obtained by subtracting the fluorescence of the blank (F_b) from the sample fluorescence (F_s). The standard error of the difference [47] is given by

$$s_d = (s_s^2 / n_s + s_b^2 / n_b)^{1/2} \quad (15)$$

where

s_s^2 = variance of sample

s_b^2 = variance of blank

n_s = number of samples

n_b = number of blanks

T A B L E 3

Minimum detectable (MDC) and determinable ($C_{\min}$) concentrations

Organic phase: methylene chloride

Aqueous phase: buffered albumin solutions

I. Measurements of MPT in organic phase

II. Measurements of PT in organic phase

III. Measurements of PT in aqueous phase after re-extraction

	C_{albumin}^0 $\times 10^4$	Blank $\times 10^9$	s_b $\times 10^9$	n	MDC $\times 10^9$	$C_{\min}$ $\times 10^9$
I	0.73	4.0	0.3	9	0.7	4.9
	5.94	16	3	9	6	44
II	0.73	11	2	18	3	25
	1.45	17.6	0.4	12	0.9	6.4
	5.94	39	4	18	8	61
III	1.45	4	1	24	2	20

MDC is defined by eq. (18). $C_{\min}$ is defined by eq. (10) in paper VII.

Relative standard deviations:

$$C_{\min} = 5\%$$

$$\text{MDC} = 34\text{--}40\% \text{ (eq. (19))}$$

At the limit of detection the approximation $s_s = s_b$ is valid and

$$s_d = s_b \times ((n_s + n_b) \times (n_s \times n_b)^{-1})^{1/2} \quad (16)$$

A t-test gives the smallest F_s which is significantly larger than F_b according to

$$F_s = F_b + t \times s_d \quad (17)$$

The minimum detectable concentration, MDC, is then obtained from the analytical calibration function:

$$\begin{aligned} \text{MDC} &= (k)^{-1} \times (F_s - F_b) = \\ &= (k)^{-1} \times t \times s_b \times ((n_s + n_b) \times (n_s \times n_b)^{-1})^{1/2} \quad (18) \end{aligned}$$

The t -value is defined by the desired confidence level (one-sided test) and the degrees of freedom used for the determination of s_b .

The magnitude of MDC is obviously determined by the slope of the calibration curve, the size of t and s_b , and the number of samples and blanks used for measurements of F_s and F_b .

The relative standard deviation (s_{rel}) at the limit of detection is given by:

$$s_{\text{rel}} = s_d / F = (t)^{-1} \quad (19)$$

MDC-values (confidence level 99%, $n_s=n_b=3$) and C_{min} -values, together with the blanks and their standard deviations obtained in extractions from albumin solutions, are given in Table 3.

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